Storage temperature determines platelet GPVI levels and function in mice and humans

Jeffrey Miles[1], S. Lawrence Bailey[1], Ava M. Obenaus[2], Molly Y. Mollica[3], Chomkan Usaneerungrueng[1], Daire Byrne[1], Lydia Fang[1], Jake R. Flynn[3], Jill Corson[1], Barbara Osborne[1], Katie Houck[1], Yi Wang[1], Yu Shen[1], Xiaoyun Fu[1,4], Jing-Fei Dong[1,4], Nathan J. Sniadecki[2,3,5], Moritz Stolla[1,4]

[1]Bloodworks Northwest Research Institute, Seattle, WA, USA
[2]University of Washington, Department of Mechanical Engineering, Seattle WA, USA
[3]University of Washington, Department of Bioengineering, Seattle WA, USA
[4]University of Washington School of Medicine Department of Medicine, Division of Hematology, Seattle, WA, USA
[5]University of Washington, Department of Laboratory Medicine & Pathology, Seattle WA, USA
Corresponding author:
Moritz Stolla, M.D.
Assistant Member, Bloodworks Northwest Research Institute
Assistant Professor, Div. of Hematology, Dept. of Medicine, University of Washington School of Medicine
Associate Medical Director, Swedish Medical Center, Blood Transfusion Service
1551 Eastlake Ave, Suite 100, Seattle, WA 98102
Phone (206) 689-6268 • Fax (844) 386-0383
Email: mstolla@bloodworksnw.org

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1) Platelets exposed to cold temperature generate contractile forces in growing thrombi under shear stress and in a static single cell assay
2) Cold exposure reduces GPVI levels in mouse and human platelets and decreases the response to GPVI agonists before and after transfusion

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ABSTRACT

Platelets are currently stored at room temperature before transfusion to maximize circulation time. This approach has numerous downsides, including limited storage duration, bacterial growth risk, and increased costs. Cold storage could alleviate these problems. However, the functional consequences of cold exposure for platelets are poorly understood. In the present study, we compared the function of cold-stored platelets (CSP) and room temperature-stored platelets (RSP) *in vitro, in vivo,* and post- transfusion. CSP formed larger aggregates under *in vitro* shear while generating similar contractile forces compared to RSP. We found significantly reduced GPVI levels after cold exposure of 5-7 days. After transfusion in humans, CSP were mostly equivalent to RSP yet aggregated significantly less to the GPVI agonist collagen. In a mouse model of platelet transfusion, we found a significantly lower response to the GPVI-dependent agonist convulxin and significantly lower GPVI levels on the surface of transfused platelets after cold storage. In summary, our data support an immediate but short-lived benefit of CSP and highlight the need for thorough investigations of this product. (NCT03787927)

Keywords: Platelet Transfusion, Cold storage, Platelet storage, GPVI, Platelet contractile forces

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INTRODUCTION

Platelets are transfused to prevent and treat bleeding. Standard platelet storage is limited to 5-7 days at room temperature (20-24 °C, RT) to limit the risk of sepsis.[1,2] As a consequence, they require additional labor and costs due to bacterial testing[3], logistical issues with maintaining an adequate supply, and shortages.

Cold-stored platelets (1-6 °C, CSP) were the standard of care in the 1960s and 1970s but were abandoned because the circulation time is severely shortened.[4] Nevertheless, storage of platelets at 4 °C has potential advantages like prolonging storage times, preventing bacterial growth, and easier transport and storage. Despite the short circulation time, CSP could be beneficial for actively bleeding trauma or surgery patients, thereby improving the availability of RT-stored platelets (RSP) for hematology and oncology patients. Numerous studies of CSP show better *in vitro* function than RSP in a wide range of assays.[5-13] These studies were mostly performed in the stored concomitant plasma and thus neglected the substantial dilution upon transfusion into humans. This dilution factor likely renders the concomitant plasma irrelevant for the stored platelet function *in vivo.* Cold exposure also elicits specific changes to the platelet cytoskeleton, such as actin filament barbed end capping, actin polymerization, and *de novo* nucleation.[14] Increased cytosolic calcium and microtubule disintegration accompany the cytoskeletal changes.[15] Combined, these phenomena mimic responses to agonists and could prime cold-exposed platelets for immediate activation at sites of vascular injury. However, persistent pre-activation could also cause desensitization and loss of efficacy. Hence, it is unclear whether cold-stored platelets can contribute to thrombus contraction under physiologic flow or generate forces on a single-cell level.[16] Previous studies have looked at platelet clot retraction in plasma and whole blood, making it challenging to dissect individual platelet and plasma protein contribution.[17-19] Few studies have investigated cold-stored platelet function after transfusion into humans.[20] These studies were predominantly published in the 1970s, and the data are inconclusive.[11,20-26] Given the unclear effect transfusion has on stored platelets and the limited post-transfusion human data available, further *in vivo* investigations of RSP and CSP are urgently needed. If CSP prove effective, one practical application is implementing a dual blood bank inventory and freeing up RSP for prophylactic transfusion and allocating CSP for therapeutic transfusions in actively bleeding patients. Whether CSP have a role in prophylactic transfusions requires further basic and clinical investigations.

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This study addresses the effects of different storage temperatures in state-of-the-art assays while controlling extracellular testing conditions. Further, we evaluate RSP and CSP's function after transfusion in humans on dual antiplatelet therapy and in untreated mice. Our findings highlight the need for post-transfusion and *in vivo* data of this product.

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METHODS

Platelet Block and Post Assay

We collected whole blood and removed platelets by centrifugation. Platelet-poor plasma (PPP) derived from fresh whole blood was used to dilute the RBC rich sample fraction to yield thrombocytopenic blood. Apheresis PRP (fresh, RSP, CSP) was added to yield a final target HCT of 40% and platelet count of 3 x 10^5/μL.

Reconstituted blood was perfused through a microfluidic channel made with polydimethylsiloxane (PDMS, Sylgard 184) with embedded microscale blocks and microposts (Figure 2A) to monitor platelet aggregation and their production of contractile forces.[16] Before testing, the microchannels were incubated with rat tail collagen type I (200 μg/mL, BD Bioscience) in 0.1 M acetic acid to support the adhesion and activation of platelets. For testing, a blood sample was flowed through the microfluidic device at a shear rate of 16,000 s^{-1} using a syringe pump (Harvard Apparatus). Platelets within the blood sample attached initially to the blocks and then aggregated to form platelet-rich plugs that bridged the 9 μm gap with each post. After 15 seconds of flow, the shear rate decreased to 500 s^{-1} to reduce aggregation rate and avoid occluding the microchannel with platelets.

The size of the aggregates and deflection of the post due to platelet forces were recorded every 2 seconds over 5 minutes using phase and fluorescent time-lapse images obtained on a Nikon Eclipse Ti-E inverted microscope with a 40X objective. The images were then quantified post-experiment using custom MATLAB scripts. The force that aggregated platelets produced was calculated using Hooke's law (explained in supplement). On day 5 of storage, this protocol was repeated with fresh thrombocytopenic whole blood derived from the same donor and PRP from apheresis platelets stored at room temperature or 4 °C.

Single Platelet Contractile Force Assay

We used a microcontact-printed, reference-free traction force microscopy approach (Beussman & Mollica, manuscript in preparation). Specifically, a polydimethylsiloxane (PDMS) stamp for microcontact printing was create by casting Sylgard 184 (prepared at a 10:1 ratio) against a silicon master with an array of circular features arranged in an

orthogonal lattice. A solution of 2.5 μg/mL Alexa-Fluor 594-conjugated bovine serum albumin (BSA) was pipetted onto the PDMS stamps' surface and allowed to adsorb for one hour. The stamp was then brought into contact with a polyvinyl alcohol (PVA) film to transfer the fluorescent BSA pattern onto the film. Subsequently, the PVA film was applied to a PDMS substrate's surface made with 95% of Sylgard 527 (prepared at a 1:1 ratio) and 5% of Sylgard 184 (prepared at a 10:1 ratio), which produced substrates with a stiffness of 12 kPa.[27] The process of microcontact printing resulted in a flexible substrate with a contiguous fluorescent coating containing an orthogonal array of circular regions that were 1 μm in diameter, 2 μm in spacing, and lacked fluorescence (termed "black dots"). The substrates with black dots were treated with 5 μg/mL VWF (Haematological Technologies) or 5 mg/mL fibrinogen (Sigma Aldrich) for one hour at RT to promote platelet adhesion.

Platelets were obtained, stored, and washed (described in more detail in the supplement). Platelets were further diluted in Tyrode's Buffer and were seeded at 2.5 x 10^7 platelets/mL onto VWF-coated black dots and at 5 x 10^7 platelets/mL onto fibrinogen- coated black dots to ensure there was separation between the platelets. After 10 minutes of incubation to allow for platelet adhesion, the black dots were gently rinsed with PBS to remove the unbound platelets. The substrates were then submerged into fresh Tyrode's Buffer for 30 minutes to allow platelets to spread and generate traction forces. Platelets on the black dots were fixed with 4% paraformaldehyde for 20 minutes and permeabilized with 0.1% Triton X-100 for 20 minutes. Platelet GPIb was labeled with a CD42b monoclonal antibody, clone SZ2 (Life Technologies), and a goat anti-mouse IgG secondary antibody (Life Technologies). Platelet F-actin was labeled with phalloidin (Life Technologies). Black dots were mounted onto glass coverslips using Fluoromount- G mounting medium (Invitrogen) and imaged with confocal microscopy using a Nikon A1R confocal microscope and a 60x oil objective. Single platelet traction forces were calculated from black dot displacement using regularized Fourier Transform Traction Cytometry. Single platelet forces were measured on N=6 donors on VWF and N=5 donors on fibrinogen. For each donor, each black dot-treatment (VWF and fibrinogen), and each condition (fresh, CSP, and RSP), an average of n = 88 platelets were measured for a total of n = 2,905 platelets included in this study.

Healthy human subjects research. The WCG IRB (Western Institutional Review Board - Copernicus Institutional Review Board Group) approved our research, and all human participants gave written informed consent. We conducted the study following the Declaration of Helsinki. All authors had access to primary clinical trial data. All authors had access to the primary clinical trial data.

Healthy human subjects demographics and recruitment.

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Fourteen subjects were enrolled in the study. Six had to be terminated early due to different reasons (Supplemental Figure 1). Eight had evaluable data for platelet transfusions. One subject did not complete the RT-storage arm because of quality control failure (Supplemental Figure 1). Overall, the mean age was 28 years (interquartile range 24-32), two subjects were female (25%), the mean height was 173cm (standard deviation ± 9.3cm), the mean weight was 70.6 kg (standard deviation ± 4.7kg), the mean body mass index was 23.8 (standard deviation ± 2.6).

In vivo survival and function of transfused mouse platelets

We collected GFP-positive whole blood from UbiC-GFP mice in ACD-A via retro- orbital bleeding. Platelet-rich plasma was isolated from the whole blood and stored at RT or at 4 °C for 24 hours before transfusion. After platelet concentrations were adjusted to 2×10^8 platelets/mL, we transfused GFP-positive platelets into WT recipients via tail-vein injection. Following transfusion, blood samples were collected from each recipient at 4, and 24 hours. These samples were gated for GFP-positive platelets. The transfused platelet population was identified as GFP+ and the endogenous population as GFP-. GPVI levels were evaluated by JAQ-1 antibody binding with an IgG-PE secondary antibody. The samples were analyzed for activation of mouse αIIbβ3 using Jon/A-PE antibody to measure the activation level. We acquired all post-transfusion recovery, and functional test data were obtained by flow cytometry.

Statistical analysis: Results are reported as mean ± standard error of the mean (SEM). Statistical significance was assessed by either paired 2-tailed Student t-test, or ANOVA with Tukey correction, as appropriate. A P value ≤ .05 was considered significant. The analyses were performed with Prism (GraphPad, La Jolla, CA).

Data sharing agreement: Contact the corresponding author for data sharing: mstolla@bloodworksnw.org.

RESULTS

Post storage platelet in vitro function

Cold exposure of platelets induces actin assembly and initiates shape change resembling the activation response to agonists like thrombin, ADP, and collagen.[14] To test if these changes affect thrombus formation and clot retraction in flowing blood, we utilized a microfluidic, collagen-coated block and post assay to quantify aggregation and contractile force generation within a growing thrombus (Figure 1A, supplemental videos).[16] In this assay, platelets in a small sample of blood are stimulated to attach and

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aggregate to form a platelet-rich plug by a local gradient in the shear rate caused by the block and post. Within a few seconds, a plug is typically large enough to bridge the gap between the block and post, and hence, the contractile force produced by the platelets can be measured by how far the post is deflected towards the block. We stored platelets collected by apheresis for five days at RT, as per current clinical practice, or at 4 °C, as previously described[28], and added them to freshly-drawn RBCs and plasma to reconstitute a whole blood sample. CSP reconstituted in fresh platelet-depleted blood samples adhered readily to the block and post and generated contractile forces that were statistically comparable to samples with freshly-drawn PRP or RSP (Figure 1B-E). CSP formed significantly larger aggregates than RSP at the early time point (60 seconds) (Figure 1B, F-G). However, after 300 seconds, the size of the CSP aggregates decreased and were statistically similar to RSP. RSP samples formed consistently smaller aggregates over time than those from fresh blood samples, and the difference between RSP and fresh was significant at 300 seconds (Figure 1H). CSP formed larger aggregates after 60 seconds, but this did not increase force generation, suggesting individual CSP were generating lower forces. To further evaluate this possibility, we utilized a novel single platelet force assay that measures single platelet contractile traction forces by quantifying the deformation of dots in a fluorescent array coated with either VWF or fibrinogen (Figure 2A). Similar to what we observed by measuring force generation by aggregates formed under flow, individual platelets on VWF or fibrinogen were statistically similar between freshly-drawn platelets, RSP, or CSP (Figure 2B-C, E-F). These results indicated that the contractile function of platelets might be independent of the storage condition. We noted that traction forces for freshly-drawn platelets, RSP, and CSP were more than two-fold higher on VWF-coating versus fibrinogen-coating. From these results, we concluded that a) CSP have a higher propensity for shear- induced aggregation than RSP and b) CSP can generate similar contractile forces as freshly-drawn platelets and RSP.

Because we observed larger aggregates under flow with a collagen-coated block and post, we tested the ability of CSP and RSP to aggregate in response to collagen. We observed a trend for increased aggregation when CSP in concomitant plasma were stimulated with collagen (Figure 2A) in agreement with previously published data.[6,28,29] However, testing platelets in their stored plasma disregards the large degree of dilution that platelets undergo upon transfusion into the entire blood volume of the recipient. Surprisingly, when testing washed platelets stimulated with collagen, the trend for better aggregation with CSP compared to RSP was reversed, and there was a clear trend for more aggregation with RSP (Figure 3B). This reversal was not seen when we stimulated platelets with ADP and arachidonic acid (Figure 3A, B). Collagen is known to activate platelets mainly through GPVI and αIIβ1. Therefore, we tested for expression levels of both receptors before and after storage. We found a significant reduction in

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GPVI levels after storage in CSP versus RSP, while αIIβ1 integrin levels remained unchanged (Figure 3C). Next, we tested for GPVI levels by liquid chromatography- mass spectrometry (nanoLC-MS/MS) on platelets obtained from a separate cohort to confirm these findings. Indeed, we found significantly lower GPVI levels in CSP than in RSP (Figure 3D). To evaluate functional differences in this cohort, we diluted platelets with separately stored plasma from the same donors. We tested for activated αIIbβ3 integrin and P-selectin expression by flow cytometry after adding the GPVI-activating snake venom convulxin. Similar to our aggregometry findings with washed platelets, we found lower integrin activation and α-granule secretion in CSP than RSP (Figure 3E).

Another critical aspect of platelet function is the ability to promote coagulation in a growing thrombus. Regardless of temperature, storage led to a trend for more thrombin generation and a shorter time to peak thrombin concentration than fresh platelets (Supplemental Figure 4), likely due to increased phosphatidylserine exposure.[28] When replacing the stored supernatant plasma with separately-stored, 4°C plasma, CSP generated more thrombin than RSP.

Cold-stored platelet function in humans

To test if the loss of GPVI during cold storage led to measurable differences after transfusion in humans, we included eight healthy human volunteers in a randomized, crossover study (described in more detail in the method section). In brief, we collected apheresis platelets for autologous transfusion. Platelets were stored at either 4 °C (CSP) or 22 °C (RSP), based on randomization. After four days of platelet storage, participants received a loading dose of ASA and clopidogrel, and 12–24 hours later were transfused the entire unit of stored platelets. We assessed the efficacy of transfusion with platelet function tests at baseline (BL), immediately before transfusion (after antiplatelet drug dosing [LD]), and three times after transfusion. A wash-out period of at least seven days between the first and the second round ensured clearance of previously transfused platelets and antiplatelet therapy; then, an identical experiment was performed with autologous platelets stored under the alternative condition (Supplemental Figure 1, Figure 4A).

Platelet transfusion parameters.

Platelet counts decreased in all subjects after platelet collection (Figure 4B). The absolute platelet counts and the corrected count increments (CCI) were significantly lower in the cold-stored platelet transfusion arm after 4 hours and 24 hours (Figure 4B, C). More information about the transfusion sequence and number of transfused platelets is provided in Supplemental Figure 2.

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Platelet Function Testing after Transfusion

Because our primary concern was that reduced GPVI levels would lead to reduced response to collagen after transfusion, we isolated and washed platelets from the transfusion recipients. We adjusted the platelet counts and stimulated with collagen. Indeed, we found significantly more aggregation after transfusion of RSP versus CSP, confirming that the loss of GPVI had measurable effects on platelet function after transfusion (Figure 4D). We tested with pathway-specific agonists to gather further information about CSP function in volunteers on dual antiplatelet therapy. Arachidonic acid showed an increase in aggregation one hour after transfusion of both products compared to post-loading dose (LD) levels, and a trend for a reduced aggregation response in the cold-stored group at the 4-hour and 24-hour time point (Figure 4 D). No significant differences between CSP and RSP or evidence of reversal of clopidogrel were seen after stimulation with ADP (Figure 4D, Supplemental Figure 3D). To confirm these findings in whole blood, we utilize the αIIbβ3 integrin activation-based, point of care assay Verify NOW. One hour after transfusion, we observed reversal of ASA with both CSP and RSP (Figure 4E). After 24 hours, ARUs (ASA reaction units) were significantly lower in the cold-stored group, suggesting the reappearance of ASA inhibition (Figure 4E). We found a trend for better platelet function after transfusion with RSP in other assays, including a shorter bleeding time at the one hour post-transfusion time point, but none of these findings were statistically significant (Supplemental Figure 3A-E). Because the bleeding time varies between individuals, we normalized the values after loading dose (Figure 4F) and calculated the change as a percentage for post transfusion time points. The majority of participants who received RSP showed improvement or no change. In contrast, 50% of the CSP group recipients showed further bleeding time prolongation (Figure 4F).

Evaluation of mouse platelet GPVI expression and function after transfusion

Our human data suggest that GPVI-levels of stored platelets remain reduced after transfusion, without recovery of function or receptor level normalization. However, the study in humans did not allow us to distinguish between transfused (stored) and endogenous platelets. To investigate this further in a living organism without dual antiplatelet therapy, we utilized a mouse model to track transfused platelets. We obtained and stored platelets from UBiC-GFP mice that have GFP fused to ubiquitin. After transfusion of GFP+ platelets into wild-type C57BL6/J mice, we obtained blood samples to assess GFP-positive platelets and their survival (Figure 5A, B). We tested platelet function by flow cytometry with an activation-specific antibody to αIIbβ3 after stimulation with the GPVI agonist convulxin. Similar to our findings in humans, the αIIbβ3 integrin activation was lower in CSP than RSP post-transfusion (Figure 5C).

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Consistent with these findings, GPVI levels on CSP were significantly lower than those of RSP after transfusion (Figure 5D).

DISCUSSION

Our study is the first to identify cold-stored platelets as force-generating contributors in growing thrombi under flow and on a single-cell level. Utilizing clot retraction assays, other groups have looked into the function of CSP and reported contradictory results.[8,30-32] Plasma factors like thrombin or fibrin generation likely obfuscated the contribution of platelets in these studies. Also, conventional clot retraction assays do not take adhesion and thrombus growth under flow into account. Interestingly, in our assay, the thrombus size was significantly larger with CSP than with RSP, but we did not observe more force generation. This finding could indicate that the cold-induced αIIbβ3 pre-activation effectively mediates larger, shear-resistant, fibrinogen-dependent platelet to platelet aggregates instead of passive agglutination. However, it is unclear why this was not followed by increased thrombus contraction strength. Our studies' advantage is that we tested platelets with minimal storage-related plasma factors and on a single platelet level. However, the spread of data, especially for fresh and cold- stored platelet force generation, is remarkable and highlights the need to better understand donor to donor variability.

Our crossover study in healthy humans shows that CSP show an immediate, ASA-reversing effect after transfusion in healthy humans. While the impact of RSP lasted over 24 hours, the inhibition re-appeared 24 hours after cold-stored platelet transfusion. This reappearance of inhibition with CSP indicates that endogenous platelet production (48 hours after antiplatelet drug dosing) was not sufficient to overcome the effect of dual antiplatelet therapy, and therefore also serves as an internal "no transfusion" control. We doubt the lower platelet count in the recipient after cold-stored platelet transfusion *per se* was responsible for this effect because we observed this phenomenon in whole blood and washed platelets with adjusted platelet counts. A pilot trial in cardiac surgery patients did not find significant chest tube output differences between RSP and CSP (both stored in PAS-E instead of 100% plasma).[26] Storing CSP in PAS-E only led to a small and non-significant reduction in GPVI levels.[33] Taken together with our study results, this may suggest that using PAS-E is a preferable storage solution for CSP, although more studies in humans are needed to investigate this further. One previous study tested 100% plasma-stored CSP in healthy human subjects on aspirin and clopidogrel. The authors stored platelets for a shorter period (72 hours) and used the bleeding time as the only readout.[34] Unsurprisingly, and very similar to our absolute bleeding time results, they did not observe any significant differences between CSP and RSP. In our study, 50% of the CSP recipients showed a bleeding time prolongation after transfusion, which could hint at a possible disadvantage of this

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product. Besides apparent efficacy in overcoming some antiplatelet therapy effects, none of our different post-transfusion assays show any advantage of CSP relative to RSP at any time point after transfusion. These results contradict ours and others previously published *in vitro* results.[5-13]

In fact, our data show detrimental effects of cold storage. GPVI receptor levels on CSP were reduced, and we observed diminished responses to GPVI agonists pre and post-transfusion in humans and mice. Our group and others recently observed more collagen-induced aggregation with CSP than RSP with concomitant plasma. It is unclear how the stored plasma supernatant improves GPVI function in CSP. There are reports that the GPVI expression levels vary approximately 1.5 fold between healthy human donors.[35] Its function is preserved even with much lower levels than observed in our study.[35] However, the authors tested fresh and otherwise functional platelets at optimized conditions. The loss of GPVI in our study has to be seen in the platelet storage lesion context. Other unfavorable storage-related factors lead to dysfunctional platelets, and any loss of GPVI is likely exacerbated.[35,36] In our human study, the subjects' endogenous platelet population was rendered dysfunctional by dual antiplatelet therapy. Hence, the antiplatelet therapy reduced the ability of the endogenous platelets to overcome the combined effect of storage lesion and loss of GPVI in transfused platelets. Nevertheless, our findings are relevant because CSP are intended to be transfused to actively bleeding patient populations with known endogenous platelet defects, like surgery patients after cardiopulmonary bypass and trauma patients.[37] We were able to reproduce our findings of decreased GPVI mediated platelet function with CSP in a mouse transfusion model without antiplatelet therapy using the more potent agonist convulxin to stimulate platelets after transfusion. Beyond αIIbβ3 integrin activation, CSP generated more thrombin than RSP, likely mediated by phosphatidylserine positive, procoagulant CSP and microparticles. When procoagulant CSP interact with vascular injury sites, they could promote coagulation locally. Evidence supporting this mechanism has been reported by other groups before.[8,13] This provides a possible alternative mechanism for how CSP could compensate for platelet receptor deficiencies in actively bleeding patients.

In the contractile force flow assay with a collagen-coated block and post, we did not see significantly different forces between RSP and CSP even though we replaced most storage plasma with fresh plasma. The presence of VWF and its ability to adhere to collagen and platelets likely helped to overcome the effect of low GPVI levels on CSP in this assay. Whether the same is true in actively bleeding humans remains to be investigated. This mechanistic difference could explain the lack of force increase in CSP, despite larger aggregates, although this warrants further investigation. It is currently unclear how platelets lose GPVI from their membrane during cold storage. Previous studies show that it can be cleaved by ADAM 10/17 or taken up by endocytosis.[38-40] One

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study observed cleavage of GPVI during RT storage.[41] Enzymatic activity is likely reduced at 4 °C, making cleavage as the primary mechanism unlikely. In a previous study, we saw more microparticles in CSP than RSP.[28] This suggests that loss of GPVI by membrane microvesicles may be responsible for the current study's findings. While we show GPVI levels are essential for CSP function, we make no claim that loss of GPVI affected platelet clearance. Other groups have identified different CSP clearance mechanisms.[42-44]

In summary, our study is the first to provide a thorough assessment of a) the contractile function, b) the pre- and post-transfusion function of cold-stored platelets compared to the current clinical standard. Importantly, clinical trials are required to test the efficacy of CSP in actively bleeding patients.

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Author contributions J.M. recruited subjects, performed apheresis collections, performed experiments, analyzed data, and helped write the manuscript, S.L.B., A.M.O., M.Y.M., C.U., D.B., J.R.F., L.F., K.H., and performed experiments and analyzed data and helped writing the manuscript, J.C., and B.O. recruited subjects, performed apheresis collections, and performed platelet transfusions, Y.W., and Y.S. performed experiments, X.F., J.F.D., N.J.S. designed experiments, analyzed data, and helped write the manuscript, M.S. designed the study, reviewed and analyzed data, and wrote a first draft of the manuscript.

Conflict of interest: N.J.S. is a co-founder, board member, and has equity in Stasys Medical Corporation. He is also a scientific advisor and has equity in Nanosurface Biomedical Inc. M.S. received research funding from Cerus. All other authors have no conflict of interest to declare.

References

1. FDA. Fatalities Reported to FDA Following Blood Collection and Transfusion Annual Summary for FY2017. 2017.

2. Dumont LJ, Kleinman S, Murphy JR, et al. Screening of single-donor apheresis platelets for bacterial contamination: the PASSPORT study results. *Transfusion*. 2010;50(3):589-599.

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3. FDA. Bacterial Risk Control Strategies for Blood Collection Establishments and Transfusion Services to Enhance the Safety and Availability of Platelets for Transfusion. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bacterial-risk-control-strategies-blood-collection-establishments-and-transfusion-services-enhance; 2019.
4. Murphy S, Gardner FH. Effect of storage temperature on maintenance of platelet viability--deleterious effect of refrigerated storage. *N Engl J Med*. 1969;280(20):1094-1098.
5. Getz TM, Montgomery RK, Bynum JA, Aden JK, Pidcoke HF, Cap AP. Storage of platelets at 4 degrees C in platelet additive solutions prevents aggregate formation and preserves platelet functional responses. *Transfusion*. 2016;56(6):1320-1328.
6. Reddoch KM, Pidcoke HF, Montgomery RK, et al. Hemostatic function of apheresis platelets stored at 4 degrees C and 22 degrees C. *Shock*. 2014;41 Suppl 1:54-61.
7. Pidcoke HF, Cap AP. Refrigerated Platelets for the Treatment of Acute Bleeding: A Review of the Literature and Reexamination of Current Standards: Reply. *Shock*. 2015;43(3):298-299.
8. Nair PM, Pidcoke HF, Cap AP, Ramasubramanian AK. Effect of cold storage on shear-induced platelet aggregation and clot strength. *J Trauma Acute Care Surg*. 2014;77(3 Suppl 2):S88-93.
9. Montgomery RK, Reddoch KM, Evani SJ, Cap AP, Ramasubramanian AK. Enhanced shear-induced platelet aggregation due to low-temperature storage. *Transfusion*. 2013;53(7):1520-1530.
10. Reddoch KM, Montgomery RK, Rodriguez AC, et al. Endothelium-Derived Inhibitors Efficiently Attenuate the Aggregation and Adhesion Responses of Refrigerated Platelets. *Shock*. 2016;45(2):220-227.
11. Becker GA, Tuccelli M, Kunicki T, Chalos MK, Aster RH. Studies of platelet concentrates stored at 22 C and 4 C. *Transfusion*. 1973;13(2):61-68.
12. Johnson L, Tan S, Wood B, Davis A, Marks DC. Refrigeration and cryopreservation of platelets differentially affect platelet metabolism and function: a comparison with conventional platelet storage conditions. *Transfusion*. 2016;56(7):1807-1818.
13. Six KR, Devloo R, Compernolle V, Feys HB. Impact of cold storage on platelets treated with Intercept pathogen inactivation. *Transfusion*. 2019;59(8):2662-2671.
14. Hoffmeister KM, Falet H, Toker A, Barkalow KL, Stossel TP, Hartwig JH. Mechanisms of cold-induced platelet actin assembly. *J Biol Chem*. 2001;276(27):24751-24759.
15. Winokur R, Hartwig JH. Mechanism of shape change in chilled human platelets. *Blood*. 1995;85(7):1796-1804.
16. Ting LH, Feghhi S, Taparia N, et al. Contractile forces in platelet aggregates under microfluidic shear gradients reflect platelet inhibition and bleeding risk. *Nat Commun*. 2019;10(1):1204.
17. Carr ME, Jr., Zekert SL. Measurement of platelet-mediated force development during plasma clot formation. *Am J Med Sci*. 1991;302(1):13-18.
18. Jen CJ, McIntire LV. The structural properties and contractile force of a clot. *Cell Motil*. 1982;2(5):445-455.
19. Cohen I, De Vries A. Platelet contractile regulation in an isometric system. *Nature*. 1973;246(5427):36-37.
20. Mack JP, Miles J, Stolla M. Cold-Stored Platelets: Review of Studies in Humans. *Transfus Med Rev*. 2020.
21. Filip DJ, Aster RH. Relative hemostatic effectiveness of human platelets stored at 4 degrees and 22 degrees C. *J Lab Clin Med*. 1978;91(4):618-624.
22. Slichter SJ, Harker LA. Preparation and storage of platelet concentrates. II. Storage variables influencing platelet viability and function. *Br J Haematol*. 1976;34(3):403-419.
23. Apelseth TK, E.K. ; Kvalheim, V.K. ; Bjerkvig C.K. ; Fosse T.K. ; Hervig, T.; Cap, A. P. ; Haaverstad, R. ; Lunde, T.F ; Kvalheim, V.K. ; Braathen, H.; Sivertsen, J. ; Apelseth, T.; Strandenes, G. Transfusion with Cold Stored Platelets in Patients Undergoing Complex Cardiothoracic Surgery with Cardiopulmonary

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Bypass Circulation: Effect on Bleeding and Thromboembolic Risk. *Transfusion*. 2017;57(Supplement S3):3-6; P3-A03A.
24. Strandenes GK, E.K. ; Bjerkvig C.K. ; Fosse, T.K. ; Hervig, T. ; Haaverstad, R. ; Kvalheim, V.K. ; Cap, A. P. ; Lunde, T.F. ; Braathen, H. ; Sivertsen, J. ; Apelseth, T. Cold stored apheresis platelets in treatment of postoperative bleeding in cardiothoracic surgery. *Transfusion*. 2016;56(Supplement S4):16A.
25. Valeri CR. Circulation and hemostatic effectiveness of platelets stored at 4 C or 22 C: studies in aspirin-treated normal volunteers. *Transfusion*. 1976;16(1):20-23.
26. Strandenes G, Sivertsen J, Bjerkvig CK, et al. A Pilot Trial of Platelets Stored Cold versus at Room Temperature for Complex Cardiothoracic Surgery. *Anesthesiology*. 2020.
27. Rodriguez ML, Beussman KM, Chun KS, et al. Substrate Stiffness, Cell Anisotropy, and Cell–Cell Contact Contribute to Enhanced Structural and Calcium Handling Properties of Human Embryonic Stem Cell-Derived Cardiomyocytes. *ACS Biomaterials Science & Engineering*. 2019;5(8):3876-3888.
28. Stolla M, Bailey SL, Fang L, et al. Effects of storage time prolongation on in vivo and in vitro characteristics of 4 degrees C-stored platelets. *Transfusion*. 2020;60(3):613-621.
29. Meledeo MA, Campbell JE, Rodriguez AC, Valenciana MV, Cap AP. Both acute delivery of and storage with magnesium sulfate promote cold-stored platelet aggregation and coagulation function. *J Trauma Acute Care Surg*. 2015;79(4 Suppl 2):S139-145.
30. Agey A, Reddoch-Cardenas K, McIntosh C, et al. Effects of Intercept pathogen reduction treatment on extended cold storage of apheresis platelets. *Transfusion*. 2020.
31. Reid TJ, LaRussa VF, Esteban G, et al. Cooling and freezing damage platelet membrane integrity. *Cryobiology*. 1999;38(3):209-224.
32. Reid TJ, Esteban G, Clear M, Gorogias M. Platelet membrane integrity during storage and activation. *Transfusion*. 1999;39(6):616-624.
33. Wood B, Padula MP, Marks DC, Johnson L. Refrigerated storage of platelets initiates changes in platelet surface marker expression and localization of intracellular proteins. *Transfusion*. 2016;56(10):2548-2559.
34. Cohn SM, Jimenez JC, Khoury L, Perez JM, Panzo M. Inability to Reverse Aspirin and Clopidogrel-induced Platelet Dysfunction with Platelet Infusion. *Cureus*. 2019;11(1):e3889.
35. Best D, Senis YA, Jarvis GE, et al. GPVI levels in platelets: relationship to platelet function at high shear. *Blood*. 2003;102(8):2811-2818.
36. Ng MSY, Tung JP, Fraser JF. Platelet Storage Lesions: What More Do We Know Now? *Transfus Med Rev*. 2018.
37. Cap AP. Platelet storage: a license to chill! *Transfusion*. 2016;56(1):13-16.
38. Takayama H, Hosaka Y, Nakayama K, et al. A novel antiplatelet antibody therapy that induces cAMP-dependent endocytosis of the GPVI/Fc receptor gamma-chain complex. *J Clin Invest*. 2008;118(5):1785-1795.
39. Bender M, Hofmann S, Stegner D, et al. Differentially regulated GPVI ectodomain shedding by multiple platelet-expressed proteinases. *Blood*. 2010;116(17):3347-3355.
40. Montague SJ, Hicks SM, Lee CS, et al. Fibrin exposure triggers alphaIIbbeta3-independent platelet aggregate formation, ADAM10 activity and glycoprotein VI shedding in a charge-dependent manner. *J Thromb Haemost*. 2020;18(6):1447-1458.
41. Hosseini E, Ghasemzadeh M, Nassaji F, Jamaat ZP. GPVI modulation during platelet activation and storage: its expression levels and ectodomain shedding compared to markers of platelet storage lesion. *Platelets*. 2017;28(5):498-508.
42. Hoffmeister KM, Felbinger TW, Falet H, et al. The clearance mechanism of chilled blood platelets. *Cell*. 2003;112(1):87-97.
43. Sorensen AL, Rumjantseva V, Nayeb-Hashemi S, et al. Role of sialic acid for platelet life span: exposure of beta-galactose results in the rapid clearance of platelets from the circulation by

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asialoglycoprotein receptor-expressing liver macrophages and hepatocytes. *Blood*. 2009;114(8):1645-1654.
44. Chen W, Druzak SA, Wang Y, et al. Refrigeration-Induced Binding of von Willebrand Factor Facilitates Fast Clearance of Refrigerated Platelets. *Arterioscler Thromb Vasc Biol*. 2017;37(12):2271-2279.

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Figure Legends

Figure 1. Platelet aggregate force and area in response to shear flow. Reconstituted whole blood samples with fresh (black triangle), RT-stored (red circles), or 4°C-stored apheresis platelets were perfused through a microfluidic device to measure the force and area of platelet aggregates formed under shear flow. (***A***) Each microfluidic channel contains multiple sets of block and post force sensors. While under flow, platelets attach and aggregate to form a plug-like structure (green) that encapsulates the block and post. Aggregated platelets are able to produce contractile forces (F) that pull the flexible post towards the rigid block. Force is calculated from displacement of the tip of the post (δ) using Hooke's law: $F = k\,\delta$, where $k = 3\pi\, E\, d^4 / 64\, L^3$, and E is the modulus of elasticity, d is the diameter, and L is the length of the post. (***B***) Representative images of platelet aggregate area 15 sec, 60 sec, and 300 sec after blood enters the channel. (***C***) Mean force of the platelet aggregates over time. N = 5, shaded regions represent SEM. (***D***) Force of the platelet aggregates at 60 seconds shown as the mean ± SEM (N = 5). (***E***) Force of the platelet aggregates at 300 seconds shown as the mean ± SEM (N = 5). (***F***) Mean area of the platelet aggregates overtime. N = 5, shaded regions represent SEM (***G***) Area of the platelet aggregates at 60 seconds shown as the mean ± SEM. N = 5, **p = 0.0032 for 4 °C and RT. (***H***) Area of the platelet aggregates at 300 seconds shown as the mean ± SEM. N = 5, and *p = 0.0103 for fresh and RT.

Figure 2. Single platelet contraction force and spread area. Apheresis platelets were washed and seeded onto flexible, PDMS substrates that were printed with an array of 'black dots' to measure traction forces and spread area of individual platelets. ***(A)*** Platelets were fixed, stained, and imaged to visualize F-actin (green), glycoprotein Ib (GPIb) (purple) and the array of black dots (orange). The magnitude and direction of platelet traction forces (blue arrows) were calculated from the displacement of the dots. ***(B)*** Traction forces were measured for fresh (gray), RT-stored (red), and 4 °C-stored (blue) platelets that were seeded onto black dot substrates coated with VWF. Violin plots show data from a representative donor for which 252 platelets were measured (117 fresh, 73 RT-stored, 62 4 °C-stored). ***(C)*** Average traction forces per platelet was measured for six donors and no statistically significant difference was observed between fresh (black triangles), RT-stored (red circles), and 4 °C-stored (blue squares).
(D) Average spread area of platelets on VWF-coated black dots was measured for each donor and no significant difference was observed between the conditions. ***(E)*** Traction

forces were measured for fresh (gray), RT-stored (red), and 4 °C-stored (blue) platelets on fibrinogen-coated black dots. Violin plots show data from a representative donor for which 242 platelets were measured (81 fresh, 84 RT-stored, 77 4 °C-stored). ***(F)*** Average traction forces and (***G***) average spread area of platelets on fibrinogen-coated back dots was measured for five donors and no significant difference was observed between conditions

Figure 3. Platelet storage temperature and response to agonists in platelet-rich plasma (PRP) and washed platelets (WP). We obtained human platelets by apheresis and used platelets either fresh (black triangles) or stored for 5 days at either 4 °C (blue squares) or 22 °C (RT, red circles). Aggregation was induced by stimulation with 5μg/mL collagen, 20μM ADP, or 0.5mM Arachidonic acid (AA), shown as maximum aggregation, mean ± SEM, n=6-7. ***(A)*** PRP (platelet-rich plasma), *left panel:* Collagen: fresh vs. RT *p=0.018. ADP: RT vs. fresh, ***p=0.0003; fresh vs. 4 °C, *p=0.0171, Arachidonic Acid: RT vs. 4 °C, *p=0.0043; RT vs. fresh, *p=0.0031. *Right panel:* representative aggregation traces ***(B)*** WP (Washed platelets), *left panel:* Collagen: fresh vs. 4 °C **p=0.0012; RT vs. fresh ***p=0.0007. ADP: RT vs. 4 °C *p=0.0323; RT vs. fresh ***p=0.0002; 4 °C vs. fresh *p=0.0276. *Right panel:* Representative aggregation traces. ***(C)*** *Left panel:* GPVI levels on platelets determined by flow cytometry with fluorochrome-conjugated anti-GPVI antibody. N=7. *p=0.0235. *Right panel:* β1 integrin levels on platelets determined by flow cytometry with fluorochrome-conjugated β1 antibody. N=7. ***(D)*** Separate cohort of healthy volunteers, whose platelets were stored for 7 days at RT or 4 °C under the same conditions as described for the original cohort. GPVI levels were determined LC- MS/MS. Results are shown as fold change from baseline (fresh). N=5, **p=0.0049. ***(E)*** Platelet-rich plasma was diluted with separately-stored plasma and stimulated with 100ng/mL convulxin. We stained with antibodies against activated integrin (PAC-1) and P-selectin (Anti-CD62P). Results shown as the change in mean fluorescence intensity (MFI) ± SEM from unstimulated, N=5, p=0.0558 for PAC-1: RT vs. 4 °C,
*p=0.0449 for P-selectin: RT vs. 4 °C.

Figure 4. Post transfusion and in vivo platelet function in healthy humans.

(A) Time course of the healthy human crossover study**.** *BL:* baseline platelet test, *LD* platelet function 24h after loading dose with 325mg ASA and 600mg clopidogrel, *1h, 4h, 24h:* platelet function tests post-transfusion ***(B)*** Absolute platelet counts at different time points of the study from individuals during RT-stored PLT transfusion round (red circles), and 4 °C-stored PLT transfusion round (blue squares), shown as mean ± SEM, n=7-8, *p=0.0110 for 4h time point and *p=0.0154 for 24h time point. ***(C)*** Corrected count increments at post-transfusion time points, shown as mean ± SEM, n=7-8, *p=0.015 for 4h time point, and *p=0.0098 for the 24h time point. ***(D)*** Platelets were washed before assessment by light transmission aggregometry. Platelets were stimulated with

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20μg/mL collagen, 20mM ADP, 0.5mM arachidonic acid. Data shown as the mean ± SEM of maximum aggregation, n=7-8, **p=0.0088. ***(E)*** Platelet reactivity tested by Verify NOW for ASA (ARU, aspirin reaction units), Mean ± SEM, n=7-8, **p=0.0018. ***(F)*** Individual responses of subjects to autologous transfusion, after loading dose (LD, normalized to zero), and 1h, 4h, and 24h post-transfusion (shown as percentage change from bleeding LD value, negative values indicate shortening of bleeding time [i.e., correction of prolonged BT] and positive values indicate prolongation of bleeding time [i.e., worsening of prolonged BT]). The left panel shows responses after transfusion of cold-stored autologous units (4 °C-Stored, n=8), and the right panel shows responses after transfusion of RT-stored autologous units (RT-Stored, n=7).

Figure 5. Stored platelet post-transfusion function in mice.

(A) Outline of the mouse platelet transfusion model (details see method section). ***(B)*** Platelet *in vivo* survival after storage for 24h at either RT (red circles), or 4 °C (blue squares). N=9, **p=0.001 for 4h: RT vs. 4 °C. ***(C)*** αIIbβ3-integrin activation in whole blood 4h, and 24h after platelet transfusion with either 24h room temperature (RT, red circles) or 24h 4 °C-stored (blue squares) platelets after stimulation with 100nM convulxin, shown as mean fluorescence intensity (MFI) ± SEM of JON/A antibody binding. N=9, *p=0.032 for 4h, **p=0.012 for 24h. ***(D)*** GPVI expression 4h, and 24h after transfusion of either 24h room temperature (RT, red circles) or 24h 4 °C-stored (blue squares) platelets. Shown as mean fluorescence intensity (MFI) ± SEM of JAQ-1 with goat anti-rat IgG-PE secondary antibody. N=9, *p=0.038 for 4h, **p=0.008 for 24h.

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Figure 1

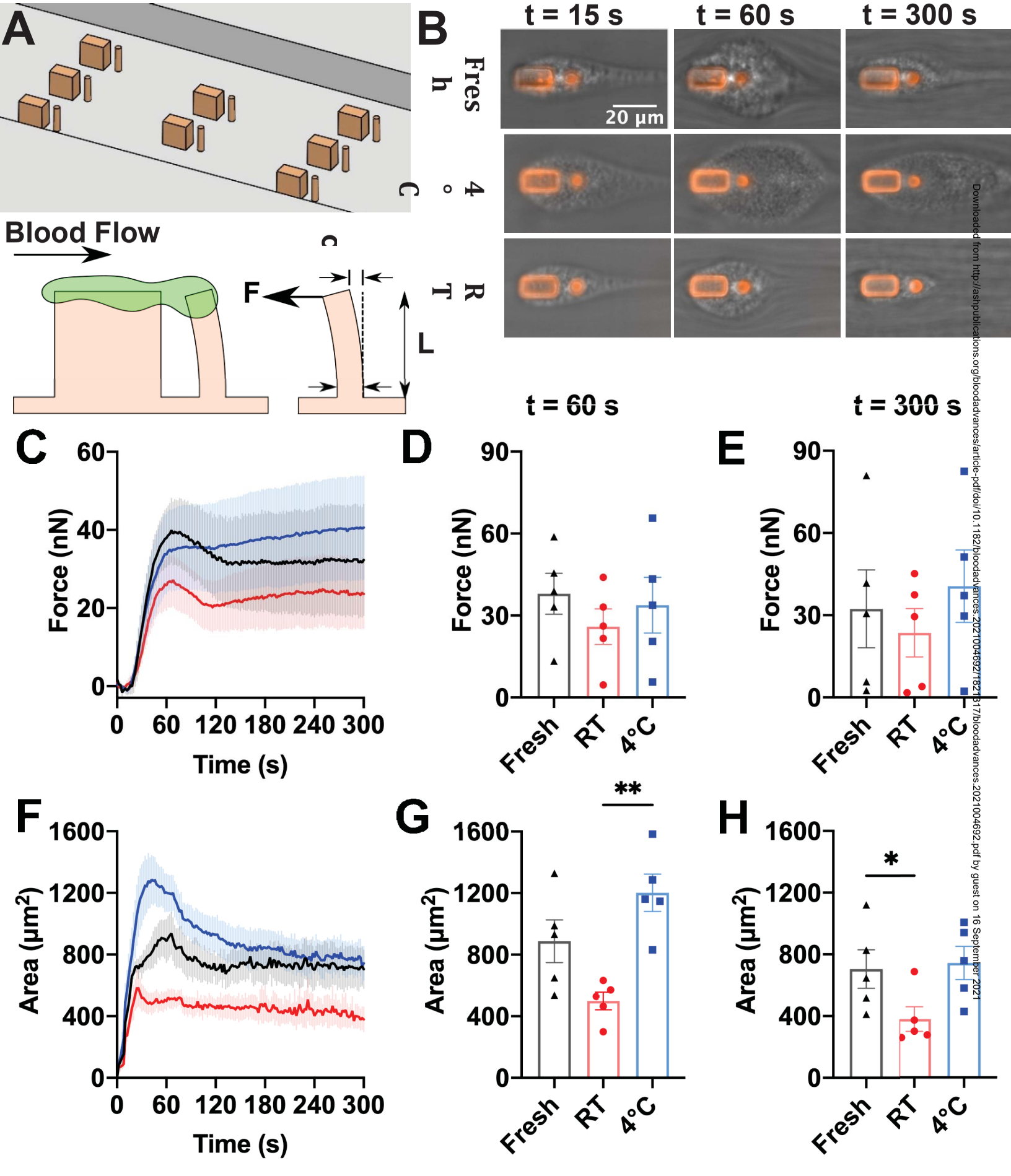

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Figure 2

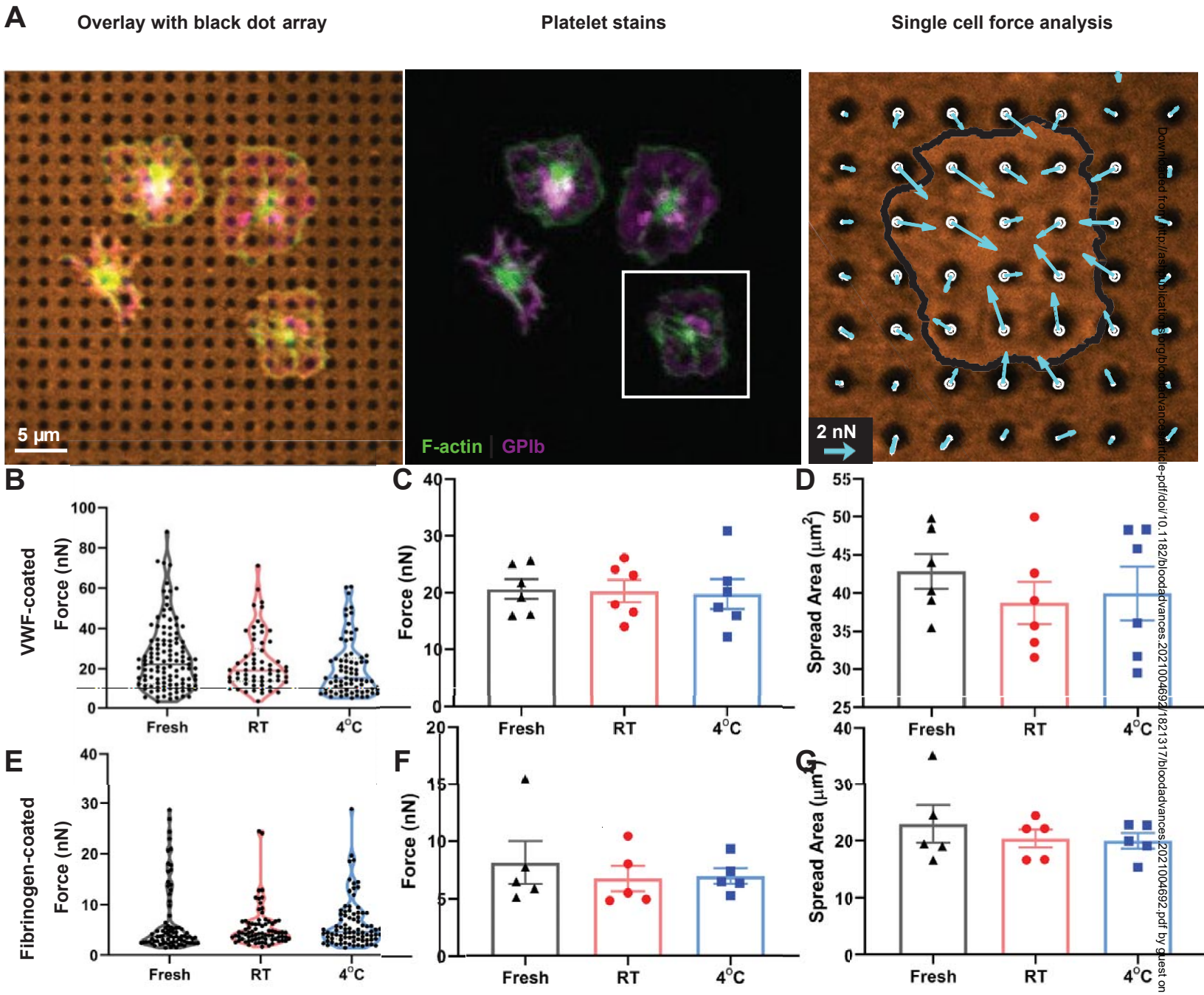

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Figure 3

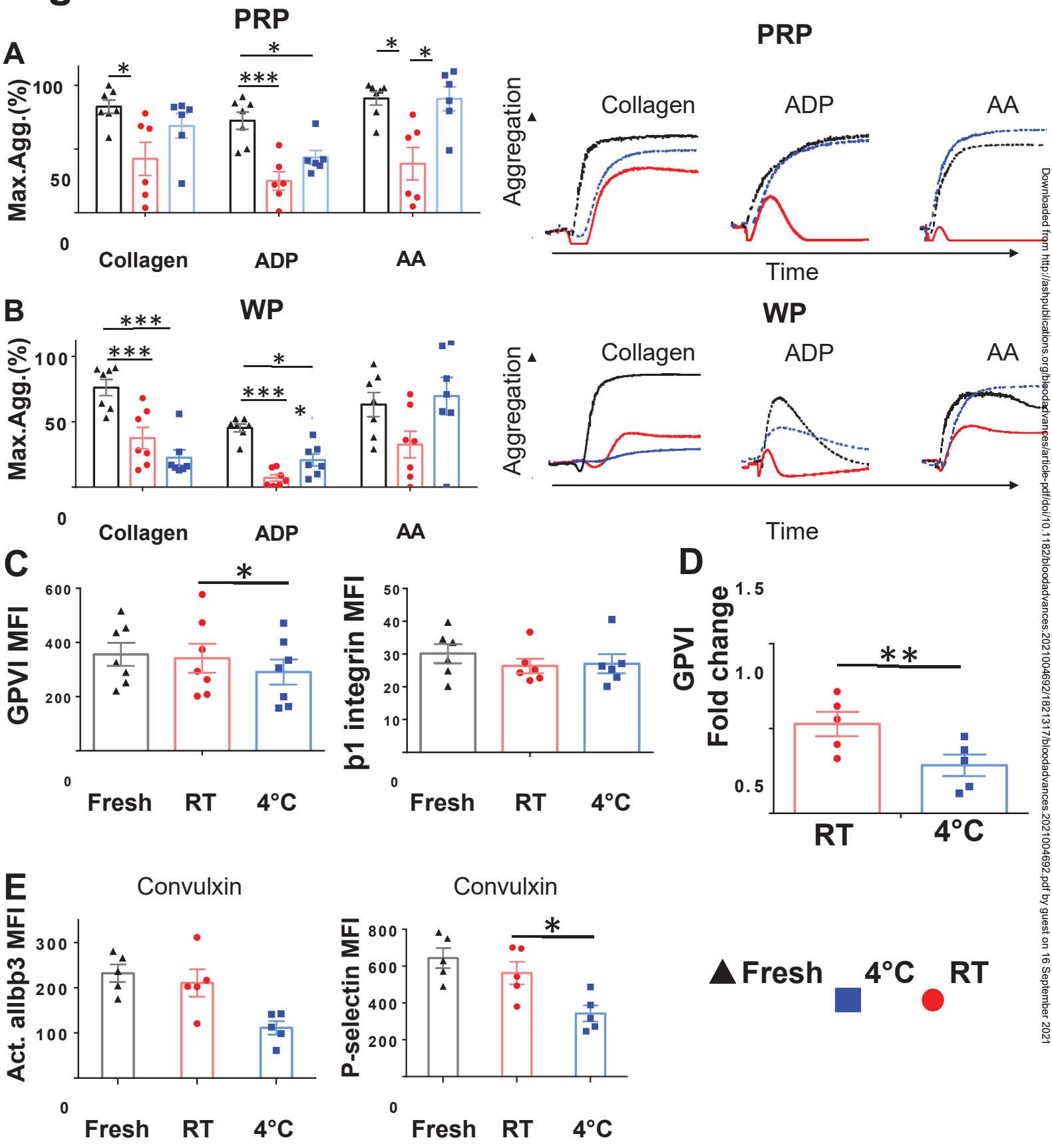

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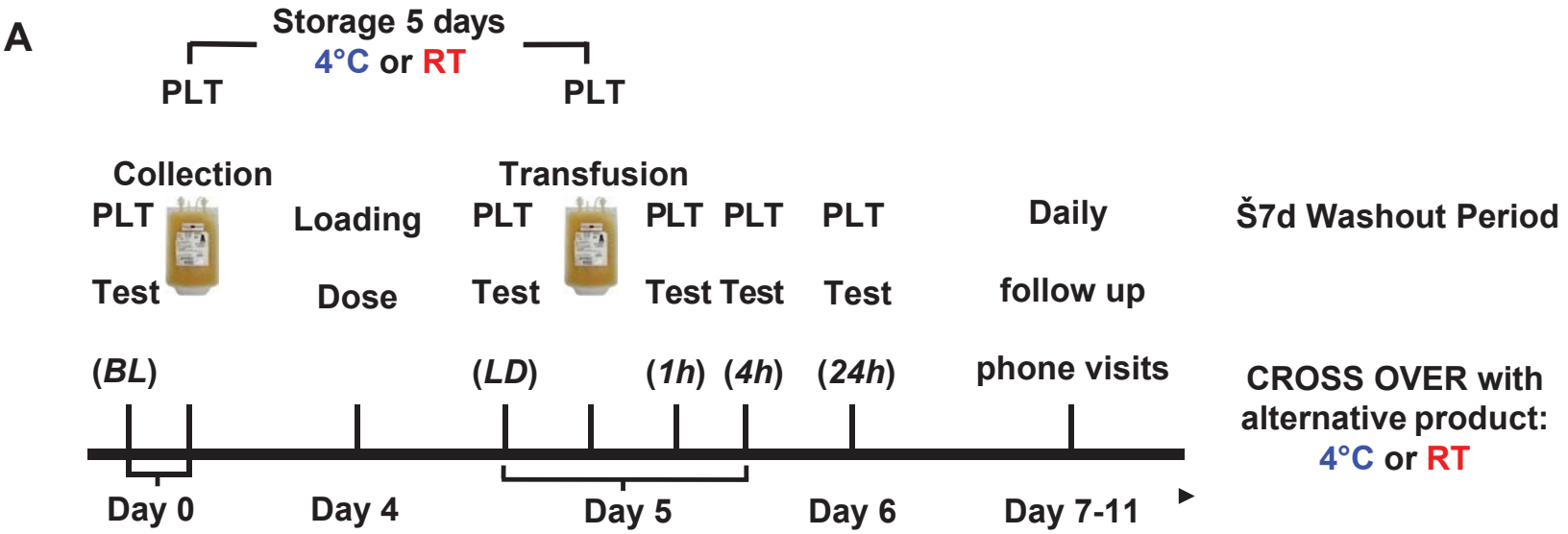
Figure 4


A
Storage 5 days
4°C or RT
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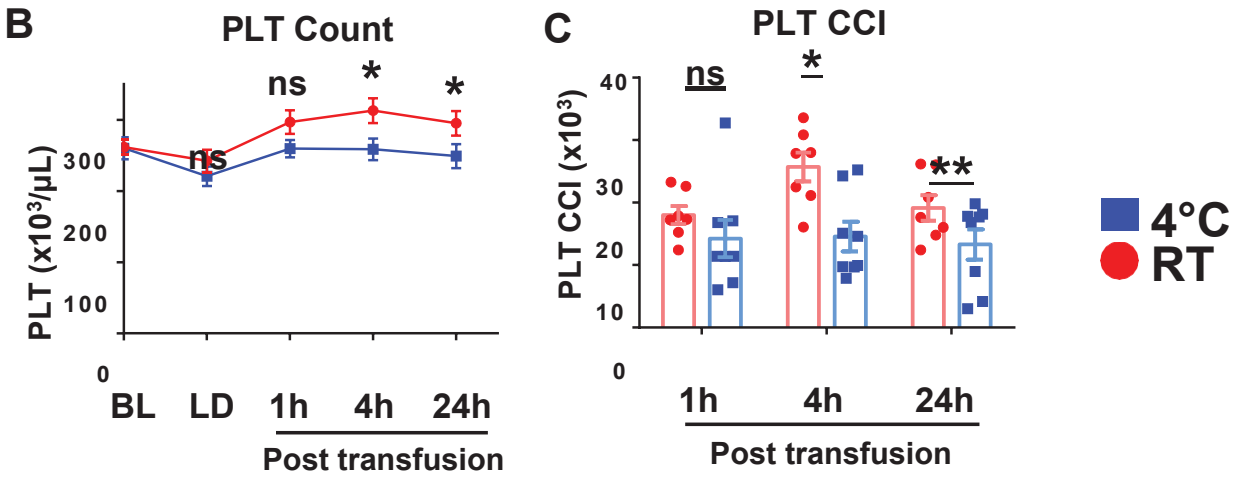
PLT
Collection
PLT
Test
(BL)
Loading
Dose
Transfusion
PLT
Test
(LD)
PLT
Test
(1h)
PLT
Test
(4h)
PLT
Test
(24h)
Daily
follow up
phone visits
Š7d Washout Period
CROSS OVER with
alternative product:
4°C or RT
Day 0
Day 4
Day 5
Day 6
Day 7-11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Post transfusion
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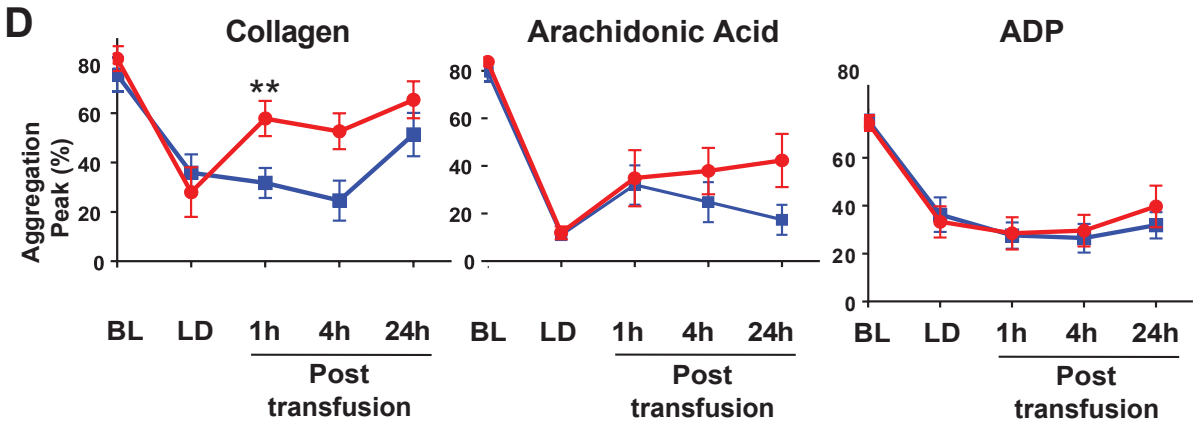
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Arachidonic Acid
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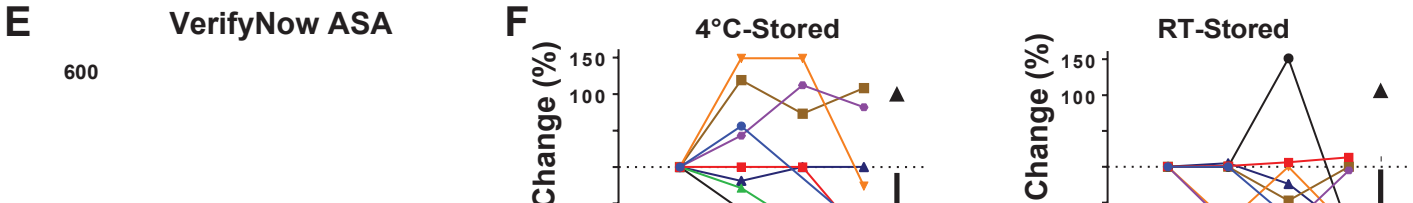
Post
transfusion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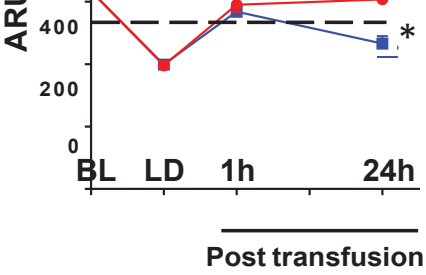
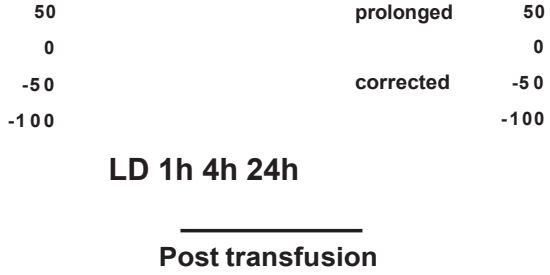
600
F
4°C-Stored
RT-Stored
Change (%)
150
100



Post transfusion



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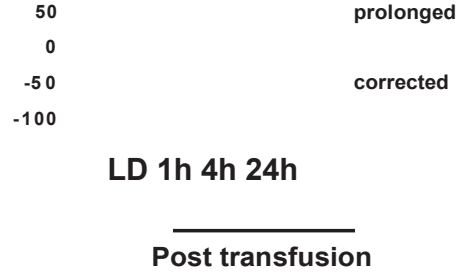
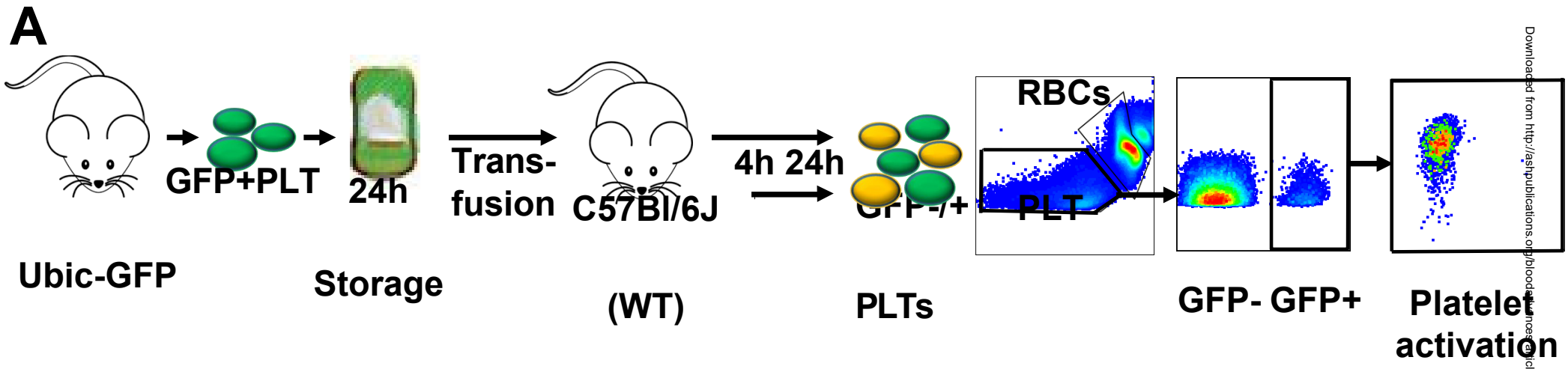
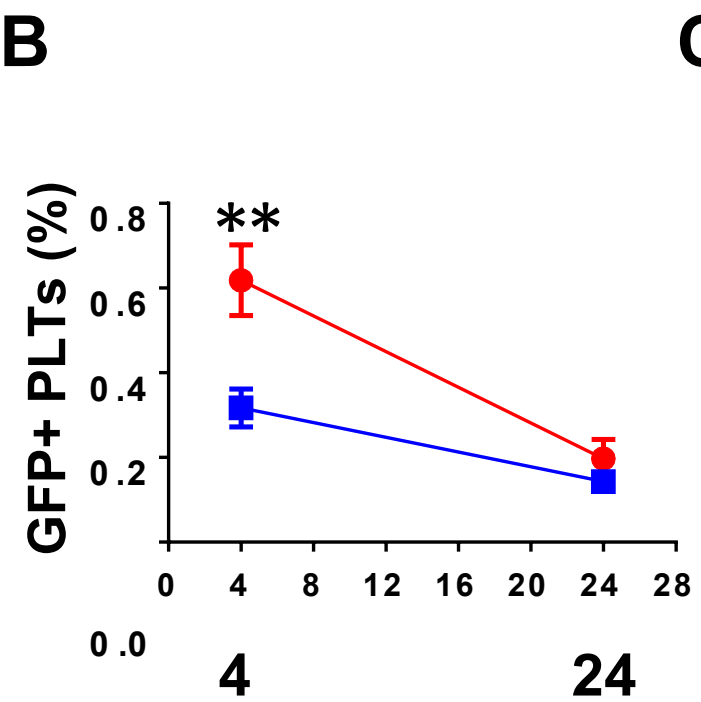


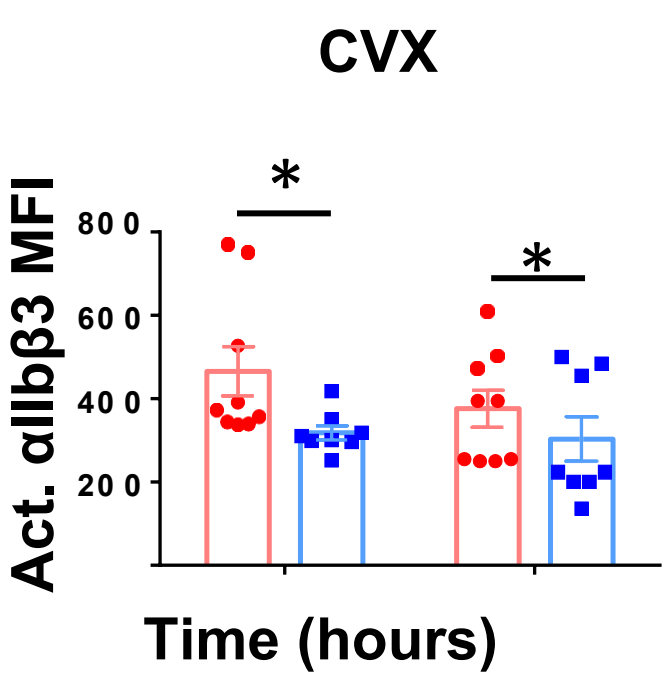
Post transfusion

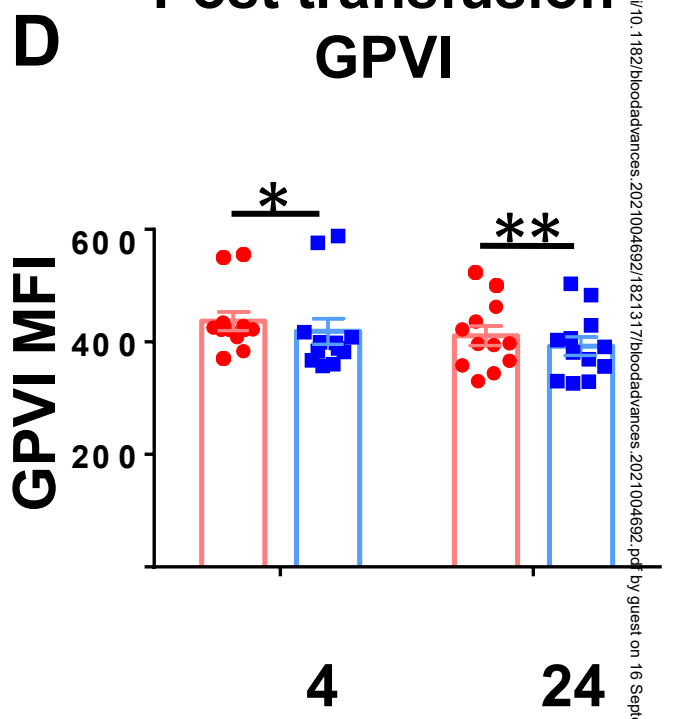
September 2021

Figure 5









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Time (hours)

4

24

Time (hours)

4°C

RT